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Chemokine Receptor Expression by Leukemic T Cells of Cutaneous T-Cell Lymphoma: Clinical and Histopathological Correlations

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Chemokine receptors expressed by normal and neoplastic lymphocytes provide an important mechanism for cells to traffic into the skin and skin-associated lymph nodes. The goal of this study was to correlate chemokine receptor and CD62L expression by circulating neoplastic T cells with the clinical and pathological findings of the leukemic phase of cutaneous T-cell lymphoma, primarily Sézary syndrome (SS). Chemokine receptor mRNA transcripts were found in the majority of leukemic cells for CCR1, CCR4, CCR7, CCR10, CXCR3, and CD62L and in 20–50% of the samples for CXCR5. In patients with SS, relatively high expression levels of CCR7 and CCR10 by circulating neoplastic T cells correlated with epidermotropism, CXCR5 expression correlated with density of the dermal infiltrate, and CD62L correlated with extent of lymphadenopathy. Of note, CXCR5 expression and a dense dermal infiltrate correlated with a poor prognosis. The chemokine receptor profile supports the concept that neoplastic T cells are central memory T cells, and that CCR10 and CD62L play a fundamental role respectively in epidermotropism and lymphadenopathy that is observed in SS.

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INTRODUCTION

The cutaneous T-cell lymphoma (CTCL) spectrum is composed of mycosis fungoides (MF), Sézary syndrome (SS), and other T-cell lymphomas that have initial manifestations in the skin (Willemze *et al.*, 2005). SS is now defined as erythrodermic CTCL with ''leukemic'' blood involvement (Vonderheid *et al.*, 2002). In our experience, each of the hematologic criteria for SS (B2 blood rating), which have been proposed by the International Society for Cutaneous Lymphomas (ISCL) defines patients with a worse prognosis compared to erythrodermic CTCL patients who lack these criteria (Vonderheid and Bernengo, 2003). Because some patients with extensive nonerythrodermic MF may also have blood findings similar to SS, we use the inclusive term ''leukemic phase of CTCL'' to include patients with SS and MF who meet the B2 blood rating defined by the ISCL (Vonderheid *et al.*, 2002).

Leukocyte migration into inflammatory sites is mediated by 8–10 kDa proteins known as chemokines, short for ''chemotactic cytokines''. These chemokines are classified into four groups, depending on the spacing between two N-terminal cysteine residues: the CXC (α-chemokine subfamily), CC (β-chemokine subfamily), XC, and CX3C chemokines (Bacon *et al.*, 2002). The ligands for these chemokines are classified by appending an R and a number to the chemokine nomenclature, that is CC, CXC, XC, or CX3C followed by R for receptor and then a number. Homeostatic receptors include CCR4, CCR7, CCR9, CXCR4, and CXCR5, and inflammatory/inducible chemokine receptors include CCR1, CCR2, CCR3, CCR5, CCR6, CCR8, CXCR1, CXCR2, CXCR3, CXCR6, and CX3CR1. Of these CCR7 and CXCR4 are highly expressed by naïve (antigen-inexperienced) T cells, and in addition, CCR2, CCR4, CCR5, CCR6, CCR10, CXCR3, CXCR5, CXCR6, and CX3CR1 become expressed by subsets of memory CD4+ T cells.

The molecular events that underlie T-cell localization within the dermis and epidermis have been reviewed (Schön *et al.*, 2003; Ebert *et al.*, 2005). Skin-homing lymphocytes are characterized by expression of cutaneous lymphocyte-associated antigen (CLA/CTAGE1). The interaction between CLA and E-selectin that is upregulated by endothelial cells of the superficial dermal plexus during inflammation causes CLA-bearing lymphocytes to roll along the vessel, and permits additional molecular interactions including chemokine-mediated conversion of low- to high-affinity integrins to occur that promote firm binding to the vessels wall and transmigration through the vessel into the dermis (diapedesis). In addition, in chronic T-cell-mediated skin diseases including CTCL, endothelial cells can develop morphologic features of high endothelial venules (HEVs), which might provide

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Abbreviations: CLA, cutaneous lymphocyte antigen; CTCL, cutaneous T-cell lymphoma; FC, flow cytometry; HEV, high endothelial venule; MF, mycosis fungoides; SS, Sézary syndrome

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another mechanism for aberrant trafficking of CD62L-bearing naïve T cells, central memory cells, and potentially neoplastic T cells of CTCL into the skin (Michie *et al.*, 1993; Lechleitner *et al.*, 1999). Chemokines secreted by dermal and epidermal cells direct T-cell migration to other regions in the skin.

The aim of this study is to assess whether chemokine receptor gene expression measured by real-time quantitative PCR (qPCR) correlates with the clinical and pathological findings of the leukemic phase of CTCL.

RESULTS

Chemokine receptor mRNA transcripts were found in the majority of leukemic cells for CCR1, CCR4, CCR7, CCR10, and CXCR3, and in 20–50% of the samples for CXCR5. (Figure 1, Table 1) The remaining gene transcripts (CCR5, CCR6) were not detected or infrequently expressed by neoplastic cells. Transcripts for CD62L were found in 18 samples.

Although hindered by cell death and nonspecific staining on stored samples, the expression mRNA transcript levels correlated well with protein expression of CXCR3 as determined by flow cytometry on nine samples. (Figure 2) This provides evidence that mRNA transcript levels in unmanipulated (i.e., activated) leukemic CTCL cells reflects the degree of protein expression in most instances as has been reported for HUT78 cell lines (Notohamiprodjo *et al.*, 2005).

Correlation to skin histopathology

The expression of several chemokine receptors and CD62L by neoplastic cells was correlated to skin histopathologic findings for a subset of 23 patients with SS on whom skin biopsy specimens taken at the time of blood sampling were available (Tables 2 and 3). For CCR1, CCR4, CXCR3, CXCR5, and CD62L, which were expressed in 15, 11, 16, 10, and 14 of the cases, respectively, the presence or absence of transcripts were compared against the dermal pattern of infiltration (perivascular *vs* band-like±interstitial infiltrate) and the degree of epidermotropism (absent *vs* basal layer *vs* diffuse). For CCR7 and CCR10, which were expressed in most cases, the cases were divided into relatively low (less than median expression values) and high (median or higher expression values).

Regarding the pattern of dermal infiltration, no correlation was found for any of the markers except for CXCR5 which was expressed by neoplastic T cells in 10 of 16 cases with a heavier dermal infiltrate compared to none of seven cases with a perivascular infiltrate (Fisher's test, $P=0.007$). With regards to epidermotropism, relatively high expression of CCR7 and CCR10 positively correlated with the magnitude of epidermal infiltration. High CCR7 expression by circulating neoplastic T cells occurred in only one of five (20%) cases without epidermal involvement, 4 of 10 (40%) cases with

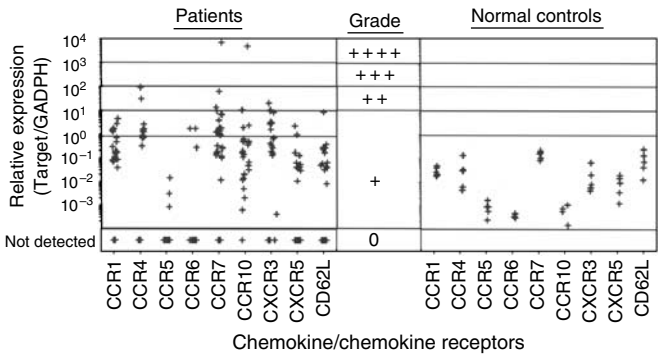

Figure 1. Real-time quantitative PCR performed on peripheral blood samples from 28 patients with leukemic phase of CTCL and 5 healthy controls. The expression levels of chemokine receptor and CD62L transcripts relative to a control gene (GADPH) are graded as defined in the legend of Table 1.

Table 1. Expression levels of chemokine receptor and CD62L gene transcripts in neoplastic cell samples from the leukemic phase of cutaneous T-cell lymphoma[1]

PT	CCR1	CCR4	CCR5	CCR6	CCR7	CCR10	CXCR3	CXCR5	CD26L
1	+	0	0	0	+	+	+	0	+
2	0	0	0	0	+	+	0	0	0
3	+	++	0	0	+	+	+	+	+
5	0	0	0	0	+	+	0	0	0
6	+	+++	0	0	+	+	+	0	+
7	0	0	0	0	+	+	+	0	0
8	+	0	0	0	+	+	++	+	0
9	+	0	0	0	0	+++	0	+	+
10	+	+	0	++	+	+	+	+	+
11	+	+	0	0	+	+	+	0	+
13	+	0	0	0	+	+	+	+	+
14	+	0	0	0	++	+	+++	0	0
15	0	0	0	0	0	++	0	0	0
16	+	0	0	0	0	++	0	+	+
17	+	+	0	0	+	+	+	+	+
18	+	+	0	0	+	+	+	0	+
20	+	+	0	0	+	+	+	0	0
21	+	+	0	0	+	+	+	+	+
22	0	0	0	0	+	+	+	0	0
23	+	+	0	0	+	+	+	0	+
24	+	+	0	0	+	+	+	0	+
25	0	0	0	0	+	+	0	0	0
26	0	++	0	0	+	+	++	+	0
27	+	+	0	0	+	0	+	+	+
28	+	++	0	0	+	+	+	+	+
29	+	0	0	0	++	+	0	+	+
30[2]	+	+	+	+	+	+	+	+	+
31[2]	0	++++	++	+++	+++	+	+	+	++++

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[1]Gene/GAPDH transcripts: 0, not detected; +, gene expression less than 10-fold of GAPDH expression; ++, gene expression 10- to<100-fold that of GAPDH; +++, gene expression 100 to<1,000-fold that of GAPDH, ++++, gene expression 1,000 or more that of GAPDH.
[2]Recently collected samples.

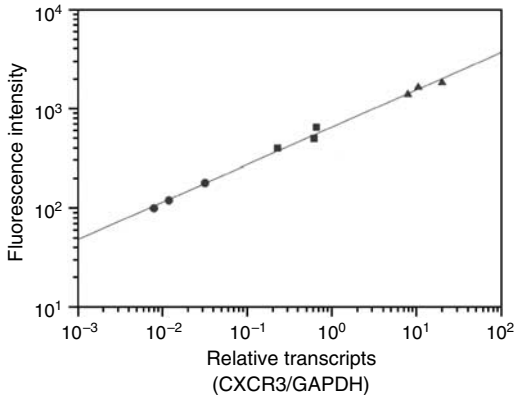

Figure 2. Flow cytometry performed on 9 blood samples of leukemic CTCL selected for low (circle), medium (square) and high (triangle) expression levels of CXCR3. A positive correlation was found between mRNA transcript levels and surface protein expression of CXCR3.

cells located along the basal layer only, and seven of eight (88%) cases with a more diffuse epidermal infiltration (χ^2 exact test, $P=0.045$). Likewise, high CCR10 expression was associated with 0 of 5 cases without epidermal infiltration, 5 of 10 (50%) with involvement of the basal layer only, and seven of eight (88%) of biopsies with diffuse epidermal involvement (χ^2 exact test, $P=0.007$).

Correlation with lymphadenopathy

The correlation between CD62L expression and lymphadenopathy is of interest, considering the key role that CD62L plays in T-cell entry into lymph nodes by initially tethering to peripheral node addressin expressed by HEVs (Table 2). CD62L transcripts were present in 14 of 18 (78%) samples obtained from patients with clinically enlarged lymph nodes *versus* two of seven (29%) samples from patients without enlarged nodes (Fisher's test, $P=0.058$). In addition, there was a positive trend between CD62L expression and extent of lymphadenopathy based on the presence of palpable lymph nodes in the cervical, axillary, and inguinal/femoral regions (χ^2, $P=0.085$). However, no difference in CD62L expression was noted between cases with palpably small *versus* large lymph nodes (Fisher's test, $P=0.524$), nor for the cases undergoing a lymph node biopsy with the specimen classified as non-effaced ($n=6$) or effaced ($n=5$ cases) nodal architecture (Fisher's test, $P=1.0$).

Although CCR7 is recognized to be the major chemokine receptor involved in recruiting T cells from the vasculature into lymph nodes, and CCR7 transcripts were found in 89% of samples from patients with SS, no correlation was found between between presence or absence of lymphadenopathy (Fisher's test, $P=1.0$), extent of lymph node enlargement (χ^2, $P=0.405$) nor maximum lymph node size (χ^2, $P=0.845$) for cases with relatively low *versus* high (defined as less than median *vs* median or higher transcripts) CCR7 expression levels.

Correlation with other measures of tumor burden

In addition to skin biopsy findings and lymphadenopathy, Sézary cell counts and serum lactic acid dehydrogenase (LDH) levels were available on all 25 patients with SS (Table 4). When the percentage of Sézary cells within the lymphocyte population was correlated to the level of chemokine receptor and CD62L transcript levels using the Spearman rank test, only CCR10 and CXCR3 were significantly correlated, that is CCR10 was positively correlated ($\rho=+0.405$, $P=0.044$), whereas CXCR3 was inversely correlated ($\rho=-0.562$, $P=0.004$). In addition, a significantly higher absolute Sézary cell count was associated with cases in which the expression of CCR10 was relatively high ($\geqslant$the median value) *versus* cases with lower values (less than the median value) of CCR10 expression (Mann–Whitney test, $P=0.008$), and conversely, absolute Sézary cell counts were significantly lower in cases in which transcripts for

Table 2. Histopathologic findings of skin specimens and lymphadenopathy in patients with SS

PT	Epidermal infiltration[1]	Dermal infiltration[2]	Maximum LN size (cm)	LN extent[3]
1	+1	+1	1	A
2	+2	+1	2	A
3	+2	+2	None	None
5	+2	+2	None	None
6	+2	+1	2	C, A, I
7	+1	+2	3	A, I
8	0	+2	None	None
9	+1	+2	2	A
10	0	+2	None	None
11	0	+1	3	A, I
13	ND	ND	3	A, I
14	+1	+2	3	A
15	+1	+2	None	None
16	+1	+2	2	C, A
17	ND	ND	3	A, I
18	+1	+1	2	A
21	0	+2	3	A, I
22	+2	+2	0	None
23	0	+1	1	A, I
24	+1	+1	1	A
25	+2	+2	2	A
26	+2	+2	0	None
29	+2	+2	6	C, A, I
30	+1	+2	1	C, A
31	+1	+2	4	C, A, I

LN, lymph node; ND, not done; SS, Sézary syndrome.
[1]Epidermal infiltration: 0=no epidermal involvement, +1=lymphocytes localized to basal layer, +2=lymphocytes at basal and suprabasal layers ± Pautrier microabscesses.
[2]Dermal infiltration: +1=perivascular involvement, +2=band like ± ⊖ interstitial involvement.
[3]Extent of lymph nodes: palpable LNs in C, cervical; A, axillary; and I, inguinal regions.

Table 3. Correlation of chemokine receptor and CD62L expression by circulating neoplastic T cells with histopathologic findings in concurrent skin specimens in 23 patients with SS

		Dermal infiltration			Epidermal infiltration			
Marker[1]	Expression	Perivascular	Band-like ± interstitial	*P*-value[2]	Absent	Basal layer	Diffuse	*P*-value[3]
CCR1	Present	6	9	0.345	5	7	3	0.090
	Absent	1	7		0	3	5	
CCR4	Present	5	6	0.193	4	4	3	0.283
	Absent	2	10		1	6	5	
CCR7	High	3	9	0.667	1	4	7	0.045
	Low	4	7		4	6	1	
CCR10	High	2	10	0.193	0	5	7	0.007
	Low	5	6		5	5	1	
CXCR3	Present	6	10	0.366	5	7	4	0.191
	Absent	1	6		0	3	4	
CXCR5	Present	0	10	0.007	3	4	3	0.748
	Absent	7	6		2	6	5	
CD62L	Present	6	8	0.167	4	7	3	0.298
	Absent	1	8		1	3	5	

SS, Sézary syndrome.
[1]Because CCR7 and CCR10 expressed in most samples, correlations made with relatively high expression levels (transcript levels ⩾ the median value).
[2]Fisher's exact test.
[3]Pearson's χ^2 exact test.

CXCR3 (Mann–Whitney test, $P=0.005$) were detected in the blood sample compared to those samples without transcripts. The most likely explanation for these observations is that circulating neoplastic T cells are predominantly CCR10+ CXCR3−. However, the correlation between CCR10 and CXCR3 transcript levels in our samples was not significant ($\rho=-0.198$, $P=0.338$). No correlation was found between serum LDH levels and transcript levels for any of the genes that were studied.

Correlation with survival

No significant correlations were found between survival curves for presence or absence of transcripts for CCR1, CCR4, CXCR3, and CD62L, nor for relatively low and high transcript levels of CCR7 and CCR10. However, the 12 cases of SS with detectable CXCR5 transcripts had a significantly worse prognosis than 13 cases without CXCR5 expression (Figure 3). Other parameters associated with an adverse prognosis for this cohort of Sézary patients included a high density of the dermal infiltrate, but not epidermal involvement, in skin specimens ($n=23$, $P=0.008$), absolute Sézary cell count ($P=0.011$), and serum LDH level ($P=0.008$). The patients' ages at the time of sample collection, presence of enlarged lymph nodes, maximum size of palpable lymph nodes, or extent of lymphadenopathy had no prognostic implications for this subset of patients. Notably, CXCR5 expression and LDH level, but not absolute Sézary cell count, retained independent significance when subjected to multivariate analysis in the Cox proportional hazards model. Furthermore, no differences were found between CXCR5-positive and CXCR5-negative subsets for patients' median ages, absolute lymphocyte and Sézary counts, CD4/CD8 ratio, and LDH level. It is therefore possible that the previously noted correlation between CXCR5 expression and degree of dermal infiltrate, another parameter with prognostic importance, may provide an explanation for the association of CXCR5 expression with prognosis.

DISCUSSION

The study provides initial evidence that circulating neoplastic cells of patients with advanced CTCL express chemokine receptor message using real-time qPCR. Unfortunately, the quality of the stored cells precluded concurrent measurement of surface protein expression in most cases. Therefore, these expression profiles ideally should be validated on freshly isolated T-cell subsets that typically contain neoplastic cells such as CD4+CD26− (Bernengo *et al.*, 2001; Sokolowska-Wojdylo *et al.*, 2005b; Fierro *et al.*, 2005b), or neoplastic cells identified by an anti-T-cell receptor Vβ antibody (Gorochov *et al.*, 1995; Morice *et al.*, 2006), or CD158/KIR3DL2. (Poszepczynska-Guigne *et al.*, 2004). Nevertheless, the fact that we performed qPCR on cases of CTCL that had high blood tumor burdens and the observation that a strong correlation exists between chemokine receptor CCR7, CCR10, CXCR3, and CXCR5 mRNAs and surface protein in CTCL-derived HUT78 cell line suggests that our findings at the gene expression level has relevance in terms of surface protein expression except when the gene expression level is quite low. (Notohamiprodjo *et al.*, 2005) Accordingly, our findings will be discussed in the context of the surface expression of corresponding proteins detected in skin lesions or blood by immunohistochemistry or flow cytometry (FC)

Table 4. Laboratory studies in patients with the leukemic phase of CTCL

PT[2]	A G	DIAG	ABSLYM (K/L)	SZ (%)	ABSSZ (mm^3)	CLONE	CHR	CD3 (%)	CD4/CD8 (ratio)	CD4+7− (%)	CD4+26− (%)	Vβ (%)	LDH Index[6]
1	68 F	SS	1.19	30	357	+ (S)	+	63	47/13 (3.6)	35	11	31	0.81
2	44 F	SS	3.13	61	1,909	− (P)5	−[5]	94	83/9 (9.2)	ND	ND	ND	0.95
3	82 M	SS	3.15	34	1,071	+ (S)	+	60	50/8 (6.3)	56	53	ND	0.69
5	73 F	SS	6.27	62	3,889	+ (P)	+	85	72/13 (5.5)	19	48	NR	0.78
6	69 F	SS	4.00	21	840	+ (P)	+	88	78/8 (9.8)	72	65	72	1.35
7	69 M	SS	9.83	24	2,359	+ (S,P)	−	95	94/1 (94)	64	92	90	0.88
8	79 F	SS	3.42	42	1,438	+ (S)	+	77	67/13 (5.2)	ND	ND	ND	1.18
9	80 M	SS	6.72	84	5,645	− (P)	+	95	94/1 (94)	15	75	79	2.53
10	60 F	SS	1.94	27	525	+ (P)	+	82	75/5 (15)	1	0	80	0.89
11	41 F	SS	3.44	53	1,823	+ (S)	+	94	91/0.4 (>100)	30	92	59	2.23
13	69 F	SS	6.32	22	1,391	+ (S,P)	+	77	75/1 (75)	23	61	66	1.15
14	71 M	SS[3]	1.72	35	660	+ (S)	+	46	80/5 (16)	38.6	22.9	ND	1.34
15	69 M	SS	1.67	71	1,189	+ (S)	+	85	76/6 (12.7)	64	62	48	0.84
16	85 F	SS	18.35	67	22,011	+ (P)	+	98	93/5 (18.6)	2	91	NR	1.71
17	80 M	SS[3]	19.49	53	10,330	+ (P)	+	98	91/6 (15.2)	11	91	83	1.23
18	83 F	SS	1.33	37	492	+ (P)	−	72	65/2 (37.5)	14	37	28	1.37
20	74 F	MFT	3.61	32	1,156	+ (P)	+	94	86/8 (10.8)	81	86	78	1.77
21	54 M	SS	2.13	50	1,066	+ (S,P)	+	91	85/5 (17)	78	74	75	0.92
22	70 F	SS	30.63	50	15,314	+ (S)	+	97	96/0.3 (>100)	95	93	NR	2.95
23	31 F	SS	0.77	50	385	− (S,P)5	−[5]	88	82/8 (10.3)	ND	ND	ND	0.93
24	60 F	SS	1.72	46	789	+ (S)	+	88	84/3 (28)	81	78	78	2.09
25	86 F	SS	60.91	64	37,901	+ (S)	+	98	99/0.4 (>100)	93	99	97	2.76
26	73 F	SS	4.74	68	3,225	− (P)	+	73	79/10 (7.9)	ND	ND	ND	1.13
27	72 F	MFPQ	5.76	28	1,614	− (S)	+	95	84/7 (12)	16	81	46	0.76
28	45 F	PTL[4]	1.25	15	188	− (P)	+	16	70/4 (17.5)	ND	ND	ND	0.66
29	52 M	SS	102.22	91	93,020	+ (S)	+	99	98/0.4 (>100)	67	99	97	3.09
30	66 M	SS	3.14	26	816	− (P)	ND	75	86/3 (28.7)	75	69	ND	1.27
31	62 M	SS	4.28	56	2,399	+ (P)	+	97	8/2 (49)	76	82	ND	1.63

ABSLYM, absolute lymphocyte count; ABSSZ, absolute Sézary cell count; AG, age and gender of patient; CLONE, molecular evidence of a T-cell clone by Southern blot (S) or PCR (P); DIAG, clinical diagnosis; LDH, lactic acid dehydrogenase; SZ, Sézary cells/100 lymphocytes.
[1]Diagnosis of Sézary syndrome rendered if erythrodermic CTCL and at least one criterion for leukemic blood involvement per recommendations of the ISCL. (Vonderheid *et al.*, 2002).
[2]Cases 4, 12, and 19 excluded because GADPH failed to amplify.
[3]Sézary syndrome preceded by MF (secondary SS).
[4]Cutaneous plaques and nodules with histologic features suggestive of Kimura's disease; abnormal T cells in blood with CD2+CD3−CD4+CD7−CD8− immunophenotype.
[5]Features of erythrodermic CTCL with abnormal cells in the blood by FC, later fulfilling criteria for SS; negative for T-cell clone at time of cell collection, positive later in course.
[6]LDH index=LDH value/top normal for laboratory reference range.

reported by others (Jones *et al.*, 2000; Lu *et al.*, 2001; Ferenczi *et al.*, 2002; Kallinich *et al.*, 2003; Yamaguchi *et al.*, 2003; Ishida *et al.*, 2004; Notohamiprodjo *et al.*, 2005; Sokolowska-Wojdylo *et al.*, 2005a, b; Fierro *et al.*, 2006; Narducci *et al.*, 2006; Yagi *et al.*, 2006).

CCR1

CCR1 transcripts were detected in 74% of the cases assessed in the present studies. Interestingly, surface expression of the CCR1 protein was not demonstrated by FC on circulating neoplastic cells in 12 cases of SS, recently reported by Narducci *et al.* (2006) and could not be detected in tumor cells infiltrating into the skin in 12 cases of MF studied by Kallinich *et al.* (2003). Thus, a discrepancy appears to exist between CCR1 mRNA expression in our samples and surface protein expression reported by others.

Although CCR1 gene transcription levels in neoplastic T cells of our cases were often increased relative to normal

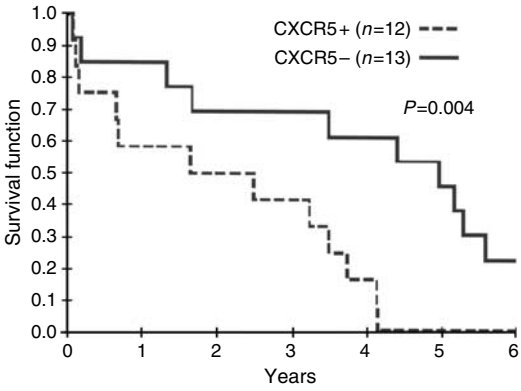

Figure 3. Survival curves for 25 patients with SS. The prognosis is worse when circulating neoplastic cells express transcripts for CXCR5 compared to CXCR5-negative cases.

lymphocytes (Figure 1), the transcription levels were relatively low compared to most other chemokine receptors. It is plausible that CCR1 expression levels in neoplastic T cells are not high enough to yield surface protein levels that are detectable by immunohistochemistry or FC. Alternatively, post-translational processing of mRNA may interfere with surface expression of the CCR1 protein or there may be rapid clearing of CCR1 protein from the cell surface.

CCR4

The results for CCR4 indicate that transcript levels are not uniformly present in samples of SS, that is only 15 (54%) cases, but when present the level of expression exceeded that of normal subjects in all instances. (Figure 1, Table 1) Indeed, a >10-fold increase in CCR4 transcripts was detected in neoplastic cells in 13 of the 15 positive cases compared to normal cells. Nevertheless, the absence of CCR4 expression in a substantial portion of our patients was unexpected considering the fact that surface protein expression of CCR4 on neoplastic cells of SS has been consistently demonstrated in a number of studies (Ferenczi *et al.*, 2002; Sokolowska-Wojdylo *et al.*, 2005a; Fierro *et al.*, 2006; Narducci *et al.*, 2006; Yagi *et al.*, 2006). In particular, Narducci *et al.* (2006) found CCR4 protein expression to range from 31 to 100% (median, 90%) of CD4+Vβ+ neoplastic cells from 12 cases of SS studied by FC. Furthermore, Fierro *et al.*, 2006 found CCR4 to be frequently expressed on CD4+CD26− lymphocytes in SS ($n=20$, mean, 59.1%, SD, 18.6%) compared to CD4+CD26− lymphocytes in benign inflammatory erythrodermas ($n=25$, mean, 11.5%, SD, 7.4%) and healthy controls ($n=24$, mean, 16.6%, SD, 4.5%). These authors proposed that the CD4+CCR4+CD26− phenotype can be used to differentiate SS from inflammatory erythrodermas.

The reason why CCR4 transcripts were absent on a subset of our leukemic CTCL cases is unclear. It is possible that CCR4 mRNA is especially labile and prone to degradation (long-term storage may have contributed to this) or that CCR4 surface protein may have a long half-life on the cell membrane even when its gene is downregulated. Another consideration is that neoplastic cells expressing relatively high levels of CCR4 mRNA and hence proteins on the cell surface might preferentially sequester in the skin, leaving behind cells in the blood with relatively low expression levels. However, this hypothesis is unlikely, considering that Fierro *et al.* (2006) found CCR4 expression to be lower on CD4+CD26− cells elaborated from the skin lesions of Sézary patients compared to the blood.

CCR7

CCR7 transcripts were detected in 25 of 28 (89%) patients in this series, and high levels of expression (>10-fold increase over normal control values) were observed in 10 patients. Surface expression of CCR7 protein has been found in all cases of SS reported thus far (Sokolowska-Wojdylo *et al.*, 2005a; Narducci *et al.*, 2006), and like CCR4, the proportion of neoplastic cells with CCR7 protein on the surface varies among cases. For example, for patient 1 with SS reported by Sokolowska-Wojdylo *et al.* (2005a), 93% of the circulating cells expressed Vβ2, but only 46% of the cells expressed surface CCR7. In the series of SS studied by Narducci *et al.* (2006), the percentage of CCR7 on Vβ+ cells ranged from 24 to 97% (median, 82%). CCR7 surface protein also was reported to be expressed in the tumor phase of MF (Kallinich *et al.*, 2003).

CCR10

At the mRNA level, CCR10 transcripts were detected in all but one of our patients with leukemic CTCL, and CCR10 transcripts were increased >10-fold over normal levels in 23 of the 27 (82%) positive samples. In fact the average increase over normal values was greater for CCR10 than any other chemokine receptor that we evaluated.

CCR10 has recently be reported to be expressed in the skin and blood of SS. (Notohamiprodjo *et al.*, 2005; Sokolowska-Wojdylo *et al.*, 2005a; Fujita *et al.*, 2006). As with CCR7, it appears that CCR10 protein is expressed on a proportion of circulating neoplastic cells in SS. For example, in the paper by Sokolowska-Wojdylo *et al.* (2005a), 55% of the lymphocytes expressed CCR10 from a patient with nearly all neoplastic cells in the blood. Fujita *et al.* (2006) also found that the number of circulating CD4+CCR10+ cells was significantly higher in MF than controls, even during the early stages. CCR10 mRNA and surface protein are expressed by CTCL-derived HUT78 cells (Notohamiprodjo *et al.*, 2005). Therefore, the results of the present studies are in agreement with published reports that neoplastic T cells of MF/SS consistently express CCR10. It is worth noting that 11 of 25 (44%) patients in this series with a chromosomally abnormal clone in the blood had evidence of an isochromosome 17 that could potentially result in an increase in copies of CCR10 because this gene is located on the long arm of chromosome 17 (17q21.1–q21.3). However, no difference in CCR10 transcript levels was found for cases with ($n=11$) or without ($n=14$) an isochromosome 17 ($P=0.412$).

CXCR3

Evaluation of CXCR3 gene expression revealed the mRNA transcripts for this receptor was detected in 75% of the samples, and that 16 of the 21 (76%) positive cases had

transcript levels >10-fold compared to normal controls. Immunohistochemical studies have demonstrated that CXCR3 is expressed by neoplastic cells in skin lesions of MF/SS, but like CCR4, the proportion of positive cells varied considerably among in individual cases (Jones *et al.*, 2000; Lu *et al.*, 2001; Kallinich *et al.*, 2003; Yamaguchi *et al.*, 2003; Notohamiprodjo *et al.*, 2005; Yagi *et al.*, 2006).

In general, CXCR3 expression was found in less than 50% of the neoplastic cells in involved skin of MF/SS in about half of the reported cases from the literature (Jones *et al.*, 2000; Lu *et al.*, 2001; Kallinich *et al.*, 2003; Notohamiprodjo *et al.*, 2005; Yagi *et al.*, 2006). Of interest, Yagi *et al.* (2006) found CXCR3 to be expressed in more than 40% of neoplastic T cells in 20/26 (77%) cases of MF, but none of five cases of SS. In one Sézary patient, FC on the skin infiltrate confirmed that Vβ9+ neoplastic T cells expressed CCR4 but not CXCR3. Several of these studies showed that CXCR3 expression was expressed by small neoplastic cells and often lost by large neoplastic cells in skin infiltrates or lymph nodes with histologic evidence of large-cell transformation, suggesting that loss of CXCR3 might be an important factor in dissemination of the disease (Jones *et al.*, 2000; Lu *et al.*, 2001; Kallinich *et al.*, 2003; Yamaguchi *et al.*, 2003; Yagi *et al.*, 2006). CXCR3 mRNA and surface protein are highly expressed by CTCL-derived HUT78 cells (Notohamiprodjo *et al.*, 2005).

Conversely, using FC, Narducci *et al.* (2006) found CXCR3 protein to be expressed on CD4+Vβ+ neoplastic cells in 11 of 12 patients, but at low levels ranging from 1 to 52% (median, 7%). By comparison, the mean CXCR3 protein expression for normal donor cells was 35% (SD, 9%). Likewise, Fierro *et al.* (2006) found CXCR3 protein to be expressed on about 8% of CD4+CD26− cells in the blood of SS, respectively, compared to a similar percentage of CD4+CD26− cells in the blood of various benign erythrodermas. These studies indicate that circulating neoplastic cells typically express low levels of CXCR3 in SS.

CXCR5

Low transcript levels for CXCR5 were detected in 14 (50%) of the leukemic samples. This contrasts with the consistent absence of CXCR5 surface protein by neoplastic T cells in skin lesions of MF including four cases studied for CXCR5 expression by reverse transcriptase PCR and *in situ* hybridization (Kallinich *et al.*, 2003; Mori *et al.*, 2003; Notohamiprodjo *et al.*, 2005). However, high expression of CXCR5 has been detected in an involved lymph node, and CTCL-derived HUT78 cells express both surface protein and mRNA for CXCR5 (Notohamiprodjo *et al.*, 2005). To our knowledge, CXCR5 protein expression has not yet been described in SS.

CD62L

Transcripts for CD62L (L-selectin/SELL) were detected in 64% of samples, but the expression levels were low in most patients. Only two cases had levels >10-fold compared to normal controls suggesting that most neoplastic T cells may not express CD62L on the cell surface, or at best, only a low level of expression. Immunohistochemical studies on skin lesions of MF indicate that neoplastic T cells are typically deficient in CD62L expression (Wood *et al.*, 1990; Magro *et al.*, 2005), but it is unclear whether this is due to an accumulation of neoplastic cells that lack CD62L or whether CD62L is shed upon activation in the skin. (Rijlaarsdam *et al.*, 1990; Chao *et al.*, 1997) With regard to circulating neoplastic T cells, there is some evidence that CD62L surface protein is also under expressed, but direct analysis on neoplastic cells is limited (Wood *et al.*, 1990; Borowitz *et al.*, 1993). Only one case of SS has been reported in which CD62L expression was studied on Vβ+ neoplastic T cells, and in this case, 95% of the Vβ17+ neoplastic cells in the blood expressed CD62L (Hwang and Fitzhugh, 2001).

In normal individuals, the majority of CLA+ cells in the blood also express high levels of CD62L (Picker *et al.*, 1990). The loss of CD62L has been associated with T_H1-type CD4+ memory T cells (Kanegane *et al.*, 1996). Because the neoplastic cells of SS are CD4+CLA+ cells with a T_H2-polarized phenotype, one would expect CD62L to be expressed. However, in concert with the concept that T-memory cells can be classified into nonpolarized central memory T cells that co-express CD62L and CCR7 and fully differentiated effector memory T cells that have lost both surface markers but capable of performing immediate effector cell functions, loss of CD62L on neoplastic T cells might be explainable. Indeed, recent animal studies indicate that an intermediate memory T-cell population defined as CD62L−CCR7+ that exists between the classic central memory T cells and effector memory T cells (Unsoeld and Pircher, 2005). In this regard, 16 of 28 samples had detectable gene transcripts for both CD62L and CCR7 followed by CD67L−CCR7+ cells ($n=9$). These results suggest that neoplastic T cells are differentiated as central memory T cells or intermediate memory T cells, and only one case had the phenotype of effector memory T cells.

The results from the present studies indicate that variable level of gene transcription is detectable in a most cases of leukemic phase CTCL for several inducible inflammatory chemokine receptors and for CD62L. Specifically, high transcript levels were found for CCR10 (all samples), CXCR3 (75% of samples), and CCR4 (54% of samples), and moderate levels of gene message were also found for CCR7 (89% of samples), results supported by studies in the literature that involve detection of surface protein. Low transcript levels were found for CCR1 (74% of samples), CXCR5 (50% of samples), and CD62L (64% of samples), and most other studies have found an absence of protein expression for these markers. Negligible gene expression was found for CCR5 and CCR6. Thus, there would appear to be good correlation between gene expression levels and protein expression if one assumes detection of mRNA transcripts is more sensitive than detection of surface protein expression and expression can vary among cases.

Considering the important role that chemokines play in the lymphocyte trafficking, the main goal of this study was to correlate chemokine receptor and CD62L expression with clinical and pathological parameters. We limited this analysis to patients with SS to provide a uniform clinical subset. Relatively high expression levels of CCR7 and CCR10 by circulating neoplastic T cells correlated with epidermotrop-

ism, CXCR5 expression correlated with density of the dermal infiltrate with both parameters associated with an adverse prognosis, and CD62L correlated with extent of lymphadenopathy. The associations between CCR10 and epidermotropism and between CD62L and extensive lymphadenopathy are not surprising considering that CCL27/CTACK, the chemokine agonist for CCR10, is secreted by keratinocytes (Fujita *et al.*, 2006), and CD62L binds to peripheral node addressin expressed by HEVs in lymph nodes, a necessary step for entry of T cells into lymph nodes. Because HEVs may also be expressed by dermal vessels in CTCL, a potential mechanism exists for CD62L+ neoplastic cells to enter the skin (Michie *et al.*, 1993; Lechleitner *et al.*, 1999). However, because the level of gene expression of CD62L was low, the corresponding surface protein might be too low to play a substantial role in trafficking into the skin. Alternatively, adequate levels of CD62L surface protein may be expressed by neoplastic T cells to allow tethering with HEVs and subsequent entry into lymph nodes and the skin, but the protein is shed from the surface upon activation in these environments. (Rijlaarsdam *et al.*, 1990; Chao *et al.*, 1997) Finally, the associations between CCR7 and epidermotropism and CXCR5 and dermal infiltration are not as evident and warrant further discussion.

Although CCR7 may be involved in entry of lymphocytes into the skin in some circumstances (Serra *et al.*, 2004), the main function of CCR7 is to regulate T-lymphocyte egress from the skin and subsequent migration into the draining lymph node (Debes *et al.*, 2005). In this regard, it was interesting that relatively high CCR7 transcript levels (median levels or higher) did not correlate with pattern of dermal infiltrate, but did correlate to the degree of epidermal infiltration. To our knowledge, CCL19, the chemokine ligand for CCR7, is not expressed by keratinocytes so the reason for the observed correlation between high CCR7 and epidermal infiltration is unclear. It may be that CCR7+ neoplastic T cells that enter the skin readily return to the blood, leaving behind cells that remain in the epidermis. Because no correlation was apparent between expression levels of CCR7 and CCR10 ($n=25$, $\rho=0.142$, $P=0.493$), it is unlikely that the association between CCR7 and epidermotropism is an epiphenomenon. The possibility that maturing dendritic cells might be a source for CCL19 must be considered because of its suspected role in certain chronic inflammatory diseases (Kaiser *et al.*, 2005; Radstake *et al.*, 2005; Ichimura *et al.*, 2006). In nonmalignant inflammatory skin diseases, CCL19 has rarely been found on infiltrating cells including dendritic cells (Christopherson *et al.*, 2003; Katou *et al.*, 2003). However, CCL19 production by dendritic cells is particularly responsive to CD40 ligation (Vissers *et al.*, 2001), and because CD40 ligand is expressed by neoplastic T cells in MF (Carbone *et al.*, 1995; Mori *et al.*, 1997; Storz *et al.*, 2001) and to a lesser degree in SS (Kamarashev *et al.*, 1998; French *et al.*, 2005), it may be that the initial interactions between CD40L-bearing neoplastic T cells and maturing Langerhans cells in the epidermis promote CCL19 release and recruitment of additional CCR7+ neoplastic cells into the epidermis. The observation that CD40L is lost in the tumor phase of MF could provide another mechanism for the loss of epidermotropism in advanced disease (Mori *et al.*, 1997).

CXCL13, the chemokine for CXCR5, functions as an arrest chemokine in the binding of B cells to lymph node HEVs and because similar structures are present in the skin of MF lesions, CXCL13 could also play a role in skin homing (Kanemitsu *et al.*, 2005). In SS, neoplastic T cells tend to localize around vessels in the superficial vascular plexus (Diwan *et al.*, 2005). However, CXCL13 was not identified in four cases of MF studied at the immunohistochemical or molecular level by Mori *et al.* (2003). On the other hand, other investigators have shown that human monocytes/macrophages are a potent inducible source of CXCL13 in inflammatory lesions where lymphoid neogenesis occurs (Carlsen *et al.*, 2004). In view of the chronic inflammatory nature of SS, local production of CXCL13 by tissue macrophages might account for the association between CXCR5 expression by circulating neoplastic T cells and the degree of dermal infiltration in the skin.

Considering the fact that both the magnitude of cellular infiltration in the dermis of SS and the presence of CXCR5 transcripts in circulating neoplastic cells are associated with a worse prognosis, and that CXCR5 expression also correlates with density of the dermal infiltrate, we hypothesize that neoplastic T cells upon entering the skin receive growth enhancing stimulation from dendritic cells and as a consequence transiently express CXCR5 which can be detected at the gene expression level in some cells that re-enter the circulation. Other chemokine receptors that might potentially be involved in skin-homing of neoplastic T cells are CXCR3 and CD62L, but neither correlated with the pattern of dermal or epidermal infiltration in our patients.

CXCR3/CD183 is a chemokine receptor with selectivity for three CXC chemokines, termed IP10 (IFN-γ-inducible 10 kDa protein), Mig (monokine induced by IFN-γg), and I-TAC (IFN-inducible T-cell α-chemoattractant). IP10, Mig, and I-TAC are commonly produced by local cells in inflammatory lesions. In MF/SS, the epidermis provides a source of CXCL10/IP10 that is thought to play a role in the characteristic epidermotropism of neoplastic T cells (Sarris *et al.*, 1997; Tensen *et al.*, 1999). The lack of an association between CXCR3 transcript levels and epidermotropism might be related to the fact that SS is a T_H2-polarized disease and therefore IFN-γ-induced chemokines such as CXCL10 might not be contributing as much to epidermal homing compared to other chemokines such as CCL27/CTACK (via CCR10) appear to play in this setting.

MATERIALS AND METHODS

Real-time qPCR was performed on samples of peripheral blood lymphocytes from 28 patients with leukemic CTCL as defined using hematologic criteria recommended by the ISCL (Vonderheid *et al.*, 2002). Samples from three additional patients (PT4, PT12, and PT19) were excluded because the mRNA in the sample had degraded. Twenty-six samples with usable mRNA were collected by leukopheresis between November 1989 and September 1997 and stored in liquid nitrogen, and two additional samples (PT30 and PT31) were obtained from recently evaluated patients (Table 1). Twenty-five

cases had generalized erythroderma (T4 skin rating) and therefore fulfilled the ISCL definition of SS (Vonderheid *et al.*, 2002). The remaining three patients also had leukemic involvement, but had skin manifestations of extensive MF (PT20 and PT27) or primary cutaneous peripheral T-cell lymphoma with histologic features resembling Kimura's disease (PT28). Blood samples from five healthy individuals (three men, two women) between the ages of 30 and 58 years were used as normal controls. These blood samples were collected under adherence to the Declaration of Helsinki Principles after informed consent was obtained and the research was approved by the Institutional Review Board of Johns Hopkins University.

The immunophenotype of the neoplastic cells in all cases was CD3 + CD4 + CD8− with a CD4/CD8 ratio of 10 or more at the time of collection for 21 (75%) cases. Absolute absolute Sézary cell counts exceeded 1.0 K/μl in 20 (71%) of the cases, and absolute lymphocyte counts exceeding 4.0 K/μl occurred in 13 patients of which 10 had evidence of a dominant T-cell clone by Southern blot (three cases), PCR analysis of the T-cell receptor γ-chain (five cases) or both methods (two cases). Chromosome studies showed an abnormal clone in the blood for 23 (82%) cases. Taken together, four patients had one ISCL criterion for leukemic involvement, five patients had two criteria, four patients had three criteria, eight patients had four criteria and seven patients had all five criteria (Vonderheid *et al.*, 2002).

qPCR reactions were performed with use of TaqMan assay (Applied Biosystems Inc., Foster City, CA) and PCR amplifications in ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described previously (Miura *et al.*, 2002, 2004). Threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission after cleavage of sequence-specific probes by nuclease activity of Taq polymerase. Target gene expression (CCR1, CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR5, and CD62L) was assessed by qPCR using an ABI PRISM 7000 Sequence Detection System and reagents (primers, probes) purchased from Applied Biosystems Inc. as described previously (Miura *et al.*, 2002, 2004). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control gene for mRNA expression. Relative transcripts were determined by the formula: $1/2^{(CTtarget-CTcontrol)}$. Primers and probe were designed so that amplicons spanned intron/exon boundaries to minimize amplification of genomic DNA. Real-time PCR efficiencies of target genes, that is CCR1) and the reference gene (glyceraldehyde-3-phosphate dehydrogenase) were approximately equal over a concentration of 0.1–200 ng total cDNA.

Statistical analysis

Because most continuous variables did not have a normal distribution, nonparametric tests (Mann–Whitney and Kruskal–Wallis tests) were used to test for differences among two and three or more groups, respectively. Fisher's and Pearson's χ^2 exact tests were used to test categorical data in 2 by 2 and R by C tables, respectively. The Cox proportional hazards model was used to examine the relationship between prognostic factors and survival. For some continuous variables, optimal discriminate cut points were established from normal laboratory reference values, cut points used in other studies or by preliminary univariate analysis of categorized data. Only factors with statistical significance on univariate analysis were entered into multivariate analysis. Survival probabilities were calculated using the method of Kaplan–Meier and survival curves were compared using the log-rank test of Mantel–Cox. Survival was determined from time of blood sampling to the death of death or last known date alive. Censoring was not required because the status of each patient was known at the time of the analysis. Death from any cause used to define overall survival. The statistical packages used for data analysis and graphs were SYSTAT10 and SPSS 13.0 for Windows (SPSS Inc., Chicago, IL), StatXact 6 and EGRET for Windows (Cytel Inc., Cambridge, MA), and SigmaPlot 9.0 (Systat Software Inc., Point Richman, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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